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A high-throughput LC–MS/MS method for the quantitation of posaconazole in human plasma: Implementing fused core silica liquid chromatography

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ABSTRACT

A rapid and robust liquid chromatographic tandem mass spectrometric (LC–MS/MS) method for the determination of posaconazole concentrations in human plasma was validated. Posaconazole was extracted from human plasma using mixed-mode cation exchange solid phase extraction in a 96-well plate format followed by gradient separation on a fused-core Halo C18 column. The analyte and its corresponding internal standard were detected using a Sciex API 4000 triple quadrupole LC–MS/MS system equipped with a TurboIonSpray™ ionization source operated in the positive ion mode. The calibration range of the method was 5.00–5000 ng/mL using a 50 μL aliquot of plasma. The assay inter-run accuracy and precision were—4.6–2.8% and 2.3–8.7%, respectively (n = 18). The results from method validation indicate the method to be sensitive, selective, accurate, and reproducible. The method was successfully applied to the routine analysis of clinical samples with the fused-core silica columns providing excellent reproducibility for greater than 1000 injections per column.

1. Introduction

Posaconazole, marketed in the United States as Noxafil®, is a triazole antifungal compound used to treat fungal infections in highly immunocompromised patients [1]. Posaconazole inhibits fungi including fusarium, candida, aspergillus, and zygomycetes by inhibiting a fungal cytochrome P450 enzyme (lanosterol 14α-demethylase) [1,2]. Posaconazole is delivered orally and bioavailability is increased when administered with food and liquid nutritional supplements such as Boost Plus® [3–5]. Clinical trials have thus far indicated that posaconazole is safe, and is more effective at treating a wider spectrum of antifungal activity than other azoles such as fluconazole and itraconazole [6].

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) is the platform of choice for supporting regulated bioanalytical analysis because it offers superior sensitivity and selectivity over older liquid chromatography ultra violet (LC-UV) detection based methods. (A recent review is available from Xu and co-workers describing advances in high-throughput quantitative bioanalysis by LC–MS/MS [7].) The recent introduction of fused-core silica particle technology has unlocked opportunities for developing higher throughput assays [8–10]. A fused-core silica particle consists of a solid silica core (d_p = 1.7 μm) surrounded by a 0.5 μm superficially porous shell (total d_p = 2.7 μm). These fused-core silica particles have shown substantial improvement in chromatographic peak efficiencies over fully porous particles [11–13]. Specifically, the reduction in axial diffusion allows for higher flow rates to be used without detriment to chromatographic performance [14,15]. Compared to sub 2 μm particles, the fused-core particles offer similar efficiency and/or peak capacity at a fraction of the backpressure [15–17]. The ability to use higher flow rates without degrading chromatography or being pressure-limited readily translates into higher bioanalytical throughput which has recently been shown in discovery studies [16,18].

Our lab previously developed a method for the determination of posaconazole concentrations in human plasma with an analytical range of 5.00–5000 ng/mL [19]. Subsequently, this method was successfully applied to the analysis of numerous clinical studies. Although the method was proven to be rugged and robust, the 4 min run time limited the number of samples that could be analyzed in an analytical run. Therefore, the development and validation of a higher throughput method was desired. Herein we present the development, validation, and application of an updated method suitable for high-throughput routine sample analysis. The method incorporates 96-well format sample processing and uses fused-core silica particle chromatography for the determination of posaconazole concentrations in human plasma. The method offers a 4-fold improvement in speed compared to the older method [19] while maintaining equivalent accuracy, precision, and reproducibility.

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2. Experimental

2.1. Reagents, matrix, and reference materials

All reagents were purchased from Fisher Scientific (Fair Lawn, NJ) unless otherwise noted. Methanol, acetonitrile, isopropanol, and acetone were HPLC-grade, and acetic acid, formic acid, and ammonium hydroxide were ACS reagent grade. Phosphoric acid (10%) was purchased from Ricca Chemical Company (Arlington, TX). Ultra-pure water was from a Millipore Milli-Q® water system (Millipore Corporation, Bedford, MA). Human plasma (with K_2EDTA as anticoagulant) was purchased from Bioreclamation Inc. (Hicksville, NY). Posaconazole (99.4% purity) and $^{15}N_2$,^{13}C-posaconazole (95.9% purity, internal standard, IS; see Fig. 1 for chemical structure) were obtained from Schering-Plough Research Institute (Kenilworth, NJ).

2.2. Sample preparation procedure

Stock solutions were prepared at a concentration of 1.0 mg/mL in methanol and stored at 4 °C. Intermediate stock solutions (100 μg/mL each for posaconazole and IS) were prepared by diluting the stock solution 10-fold in methanol. The intermediate stocks were also stored at 4 °C. Calibration standards and quality control (QC) samples were prepared by serial dilutions using blank human plasma from the 100 μg/mL intermediate stock solution. Ten calibration standards were used to establish the analytical range of 5.00–5000 ng/mL. Five concentration levels of QC samples were interspersed over this range. Calibration standards and quality control samples were stored at −20 °C until use. Long term frozen storage stability of plasma samples was previously demonstrated for at least 105 days at −20 °C [19].

Samples were prepared by aliquotting 50 μL of plasma sample (calibration standard, QC, blank, and unknowns) and 20 μL of internal standard working solution (ISWS) into individual 96-well format dilution tubes. The ISWS (3.0 μg/mL) was prepared by diluting the appropriate amount of IS intermediate stock into human plasma. For blank matrix samples, 20 μL of human plasma was added instead of ISWS. A 250 μL aliquot of 10% phosphoric acid was then added to each sample. Sample tubes were capped and then briefly vortexed.

SPE was performed using 96-well format Waters Oasis MCX 96-well (10 mg) extraction cartridges (Waters Corporation, Milford, MA) and a Tomtec Quadra96® automated liquid handler system (Tomtec Corp. Hamden, CT). The SPE bed was initially conditioned with 300 μL of methanol followed by 400 μL of 1% formic acid. Samples were then loaded, washed with 300 μL of 1% formic acid followed by 200 μL of methanol/acetone/formic acid (50/50/1, v/v/v). Samples were eluted into a 96-well True Taper™ collection plate (Analytical Sales and Services, Pompton Plains, NJ) with 100 μL of methanol/ammonium hydroxide (95/5, v/v) and evaporated to near dryness using nitrogen and a SPE Dry Dual evaporator (Argonaut Technologies, Inc., Foster City, CA). The dried samples were reconstituted with 100 μL of water/acetonitrile/acetic acid (60/40/0.1, v/v/v) prior to being placed in the autosampler for analysis.

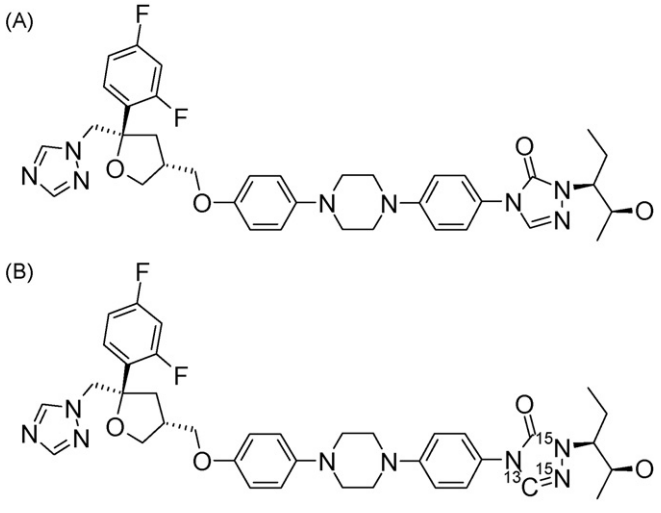

Fig. 1. Chemical structure of posaconazole (A) and the internal standard $^{15}N_2$,^{13}C-posaconazole (B).

2.3. LC–MS/MS conditions

Samples were analyzed using a Waters ACQUITY UPLC® system (Milford, MA) coupled to an Applied Biosystems/MDS Sciex API 4000™ triple quadrupole mass spectrometer (Concord, ON). Chromatography was performed using a Halo C18, 2.1 mm × 50 mm, 2.7 μm column (MAC-MOD Analytical, Inc., Chadds Ford, PA). Mobile phase A (MPA) consisted of 0.1% acetic acid in water and mobile phase B (MPB) consisted of 0.1% acetic acid in acetonitrile. Posaconazole and IS were separated from endogenous matrix components using a gradient elution. The initial mobile phase conditions (60/40, MPA/MPB) were held for 0.1 min, followed by a linear gradient from 60/40 (MPA/MPB) to 5/95 (MPA/MPB) over 0.5 min. The mobile phase composition was then maintained at 100% MPB for 0.2 min before re-equilibration to initial conditions for 0.25 min. The flow rate was 0.8 mL/min and the column temperature was set to 50 °C. The cycle time for a single analysis was 1.05 min. The autosampler weak wash was acetonitrile/water (10/90, v/v) and the strong autosampler wash was acetonitrile/isopropanol/acetone (60/30/10, v/v/v). A 2–10 μL sample aliquot achieved a system suitability requirement of S/N ≥ 10:1 at the lower limit of quantitation (LLOQ). The retention times of posaconazole and IS were both ∼0.57 min (k′ ∼ 1.3).

Samples were ionized using a TurboIonSpray® probe in the positive-ion mode. The TurboIonSpray® voltage and temperature were set to 4500 V and 500 °C, respectively. The declustering potential was 85 V and the collision energy was 47 eV. The collision gas was set to maintain an indicated pressure of $\sim 3.5 \times 10^{-5}$ torr. The mass spectrometer was tuned for unit mass resolution and data was collected using multiple reaction monitoring mode (MRM). The mass transitions used to detect posaconazole and its IS were m/z 701 → m/z 683 and m/z 705 → m/z 687, respectively, with a dwell time of 50 ms for each transition. The naturally occurring $^{13}C_2$ isotope of the IS was used to improve selectivity.

2.4. Data analysis

Data was acquired and integrated using the Applied Biosystems/MDS Sciex Analyst software package (Version 1.4.2). Data regression was performed using Watson™ Laboratory Information Management System 6.4.0.03 (Thermo LabSystems, Philadelphia, PA).

2.5. Validation requirements and acceptance criteria

The method was validated in accordance with the FDA guidance for Bioanalytical Method Validation [20–22]. Specifically, three analytical runs were processed and analyzed to assess sensitivity, reproducibility, accuracy and precision. Each analytical run contained ten calibration standards defining the analytical range ($n = 2$ at each level), two control blanks (blanks with no IS), two zero standards (blanks with IS), and quality control samples ($n = 6$ at each level).

The predefined acceptance criteria for a successful analytical run required that 3/4 of the calibration standards must have accuracy within ±15% of the nominal value (±20% at the LLOQ). Any calibration standard with a nominal value greater than ±15% is omitted from the regression. At least one standard at each of the LLOQ and ULOQ levels must meet the acceptance criteria to successfully define the analytical range. At least 2/3 of the total QC samples and at least 50% of the QC samples at each concentration level must have accuracy within ±15% of the nominal value (±20% at the LLOQ). Inter-run and intra-run mean accuracy and precision must be ±15% (±20% at the LLOQ) and ≤15% (≤20% at the LLOQ), respectively.

3. Results and discussion

3.1. Mass spectrometry

Posaconazole gives an abundant protonated molecular ion of *m/z* 701, and following collisional activation, a predominant product ion of *m/z* 683 (the loss of water). Although *m/z* 704 was the predominant protonated molecular ion for the internal standard, $^{15}N_2$,^{13}C-posaconazole, the corresponding mass transition for the naturally occurring $^{13}C_2$ isotope of *m/z* 705 → *m/z* 687 was monitored during analysis for improved selectivity. TurboIonSpray® was chosen over APCI because the resulting MRM signal was approximately 90 times more intense.

3.2. Sample extraction

The primary focus of method development was to reduce the potential for matrix effects resulting from either ion suppression or enhancement from endogenous matrix components [23–26]. Reducing the potential for matrix effects is an important step in ensuring accurate (and reproducible) concentration data for study samples that may contain different levels of matrix components [27,28]. Matrix effects can be minimized through appropriate optimization of sample preparation [26,29] and/or chromatographic parameters [30,31].

Previous reports from our laboratory suggested that C18-based SPE is not a suitable technique for the extraction of posaconazole from human plasma [19]. Our initial results using C18 SPE resulted in variable recovery that was highly dependent on the dosing formulation. In this report, we investigated the use of a mixed-mode cation exchange phase (Waters Oasis MCX) as an alternative to C18 SPE. Under acidic conditions, posaconazole should be sufficiently charged to interact with a cation exchange solid support allowing for aggressive organic washes to remove neutral and acidic interferences. Under basic elution conditions, posaconazole should be recovered with high efficiency. When coupled with C18-based chromatography, this orthogonal approach (i.e., ion exchange SPE followed by hydrophobic chromatography) should substantially reduce the potential for negative matrix effects [32].

Spiked bioanalytical standards were prepared from both drug substance and from the drug product being an intravenous dosing solution consisting of palmitoyl oleoyl phosphatidylcholine and trehalose. Following acidification with dilute phosphoric acid, samples were loaded into individual wells of a 96-well SPE plate which was then washed with an acidic aqueous solution and an acidic organic solution (see Section 2) to remove the endogenous proteins/phospholipids while retaining the analyte of interest. Different wash solutions were screened for their ability to reduce phospholipid content (monitored by summing the representative positive ion MRM transitions of *m/z* 520 → *m/z* 184 and *m/z* 496 → *m/z* 184) while maintaining high recoveries of posaconazole (Fig. 2A and B). Of the five wash solutions tested, solutions C and D provided extracts with the lowest phospholipid content (Fig. 2A, see figure caption for details). Fig. 2B illustrates the recoveries obtained using the various wash solutions; solution C resulted in a recovery of 62% whereas solution D resulted in 57% recovery of posaconazole. Solution C (methanol/acetone/formic acid, 50/50/1, v/v/v) was therefore chosen as the wash solution based on a balance between reasonable recovery and low phospholipid content.

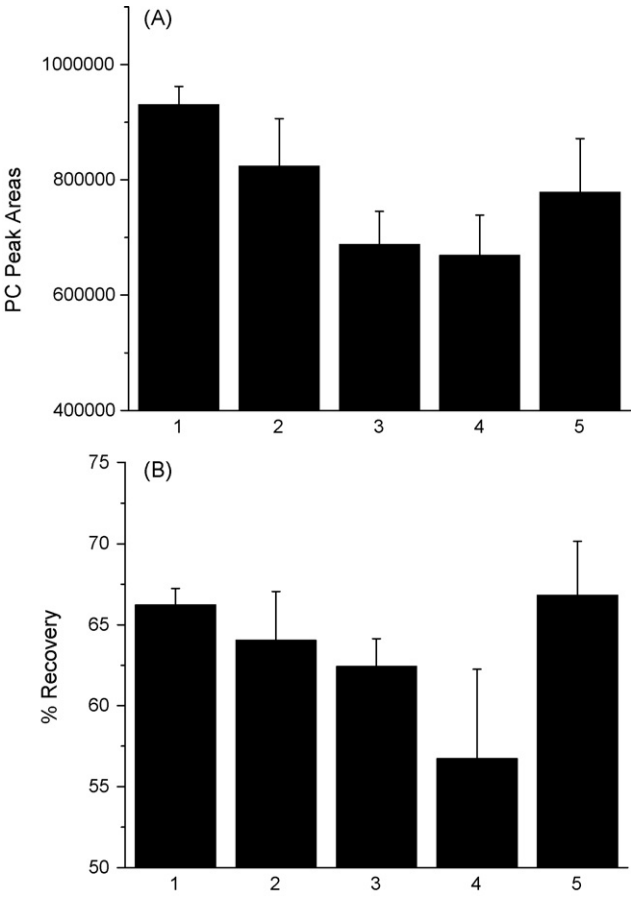

Fig. 2. The SPE wash solution is optimized for phosphatidylcholine (PC) removal (A) and posaconazole recovery (B). The PC peak areas were calculated by summing the positive-ion mode MRM transitions *m/z* 520 → *m/z* 184 and *m/z* 496 → *m/z* 184. The wash solutions were (1) methanol; (2) 50/50 acetone/methanol; (3) 50/50/1 acetone/methanol/formic acid; (4) 70/30 acetonitrile/methanol; and (5) 70/30/1 acetonitrile/methanol/formic acid. The elution solution was 90/5/5 methanol/water/ammonium hydroxide. All solutions are listed in v/v or v/v/v format. Other experimental details can be found in Section 2. Error bars represent standard deviation ($n = 4$).

A solution containing 90/5/5 methanol/water/ammonium hydroxide was used to elute the samples during the wash solution optimization experiment discussed above. To maximize recovery, the elution solution was similarly optimized and a 95/5 (v/v) methanol/ammonium hydroxide solution increased the recovery from 62% (using 90/5/5 methanol/water/ammonium hydroxide) to 80–90% (see Section 3.8). Most importantly, there was no apparent difference in recovery from plasma samples prepared from either the bulk material or formulated posaconazole (data not shown).

3.3. Chromatography

The primary goal for the development of a chromatographic method was to rapidly separate posaconazole from the remaining endogenous phospholipids in order to reduce the potential for ion suppression and negative matrix effects. A Mac-Mod Halo C18, 2.1 mm × 50 mm, 2.7 μm column was chosen because of its high peak capacity, ruggedness, and reduced analysis times [15,16]. The

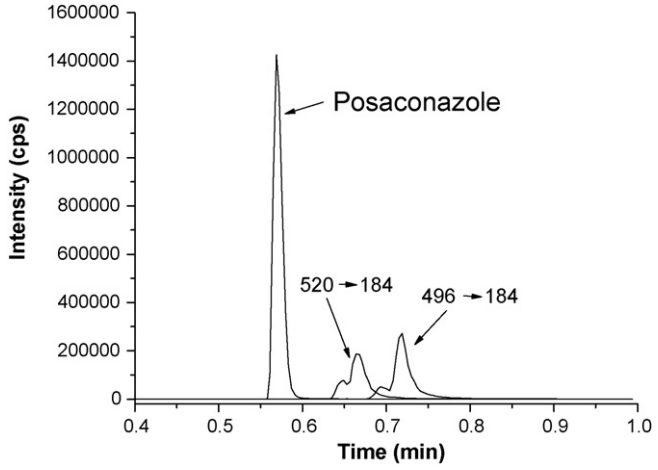

Fig. 3. Separation of posaconazole (m/z 701 → m/z 683) from phospholipids (m/z 496 → m/z 184 and m/z 520 → m/z 184) in the SPE extract. The posaconazole concentration was 4 μg/mL. The three MRM traces are overlaid for clarity. All other experimental details are listed in Section 2.

maximum mass spectrometric response was obtained using gradient elution (see Section 2) with a mobile phase consisting of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile. A column temperature of 50 °C provided optimum peak shape and peak height while maintaining a low system backpressure. Fig. 3 illustrates the separation of posaconazole (t_r = 0.57 min) from two representative glycerophosphatidylcholine esters. Monitoring the ion signal for precursor ions of m/z 184 provided adequate response to assess chromatographic resolution from these potential matrix interferences [24,31,33]. The cycle time for the LC–MS/MS method was 1.05 min, which represents an ~4× improvement over the previously published method from our laboratory [19].

Representative ion chromatograms obtained at the LLOQ are shown in Fig. 4. A 3 μL injection volume of an extracted LLOQ plasma sample (7.50 pg on column, assuming 100% recovery) gave a peak response with S/N of ~30.

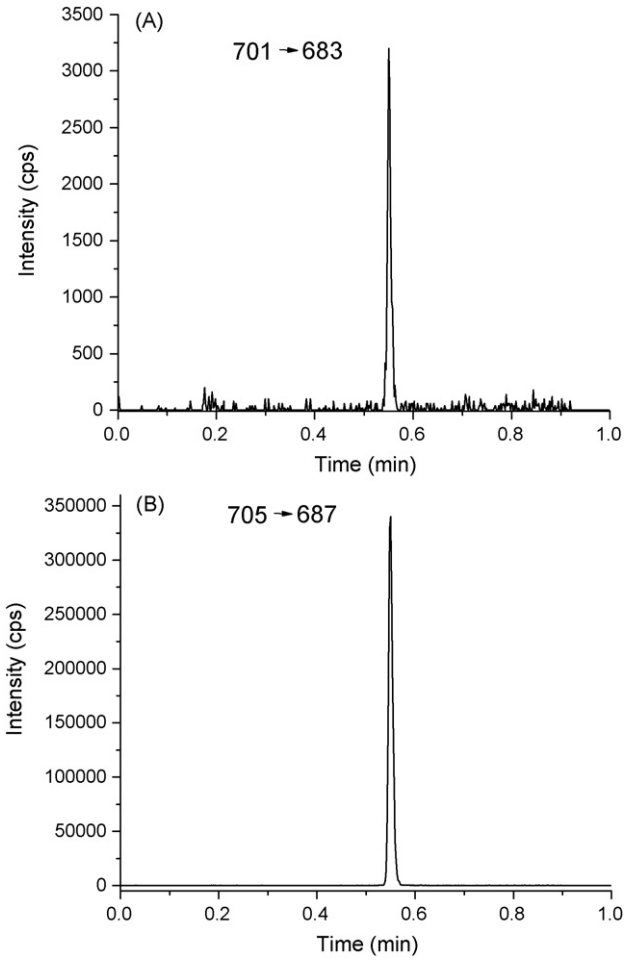

Fig. 4. Representative MRM chromatograms of an extracted 5.00 ng/mL LLOQ human plasma sample of posaconazole (A) and internal standard (B). Injection volume was 3 μL. Extraction and LC conditions are listed in Section 2.

3.4. Linearity

The peak area ratios of calibration standards were proportional to the concentration of analytes in each analytical run over the nominal concentration range of 5.00–5000 ng/mL. The calibration curves appeared quadratic and were well described by least squares regression lines. A weighting factor of 1/concentration^2 was chosen to achieve homogeneity of variance. The slopes, intercepts, and coefficients of determination (r^2) from the validation are summarized in Table 1.

3.5. Accuracy and precision

The calibration standards inter-run precision and accuracy results from the three analytical core runs are listed in Table 1. The inter-run accuracy (%DIFF) ranged from −2.5 to 3.0% (n = 6). The

Table 1
Back-calculated posaconazole concentrations in human plasma. Data was fit using a quadratic regression ($y = Ax^2 + Bx + C$) with a weighting of 1/concentration^2. y is the peak area ratio, x is the concentration of posaconazole, and A, B, and C are the calibration curve parameters. %CV: percent coefficient of variation (precision); %DIFF: percent difference from nominal value (accuracy); and S.D.: standard deviation. All concentrations are listed to three significant figures.

Concentration [ng/mL]	STD1 5.00	STD2 10.0	STD3 20.0	STD4 50.0	STD5 100	STD6 250	STD7 500	STD8 2000	STD9 4250	STD10 5000	A	B	C	r^2
Core run 1	4.76	9.59	18.7	50.6	103	243	483	2110	4200	4890	−3.2E-10	0.00138	0.000689	0.9984
	5.32	10.4	19.9	50.0	100	248	506	2080	4300	4930				
Core run 2	4.65	10.3	19.7	49.3	92.8	256	508	2080	4150	4800	−8E-09	0.00168	0.000995	0.9979
	5.43	9.68	19.6	48.3	103	248	520	2050	4300	5080				
Core run 3	4.90	9.06	18.8	49.3	99.8	256	507	2070	4240	5180	−4.2E-08	0.00162	0.00124	0.9973
	5.42	9.95	20.3	47.8	102	253	531	1970	4440	4530				
n	6	6	6	6	6	6	6	6	6	6		3		
Overall mean	5.08	9.83	19.5	49.2	100	251	509	2060	4270	4900		0.00156		
S.D.	0.35	0.50	0.6	1.0	4	5	16	50	100	230		0.000159		
%CV	6.9	5.1	3.2	2.1	3.8	2.1	3.2	2.3	2.4	4.6		10.2		
%DIFF	1.6	−1.7	−2.5	−1.6	0.0	0.4	1.8	3.0	0.5	−2.0				

Table 2
Accuracy and precision data for extracted posaconazole quality control samples in human plasma. LLOQ: lower limit of quantitation; QCH: quality control of high concentration; QCL: quality control of low concentration; QCM: quality control of medium concentration; QCML: quality control of medium-low concentration. See Table 1 for additional abbreviations. All concentrations are listed to three significant figures.

Core run	LLOQ	QCL	QCM-L	QCM	QCH
1	5.27	14.7	153	423	4230
	5.09	15.5	159	417	4250
	5.08	15.6	157	418	4030
	4.89	15.2	156	422	4250
	4.93	15.6	146	407	4120
	5.29	14.8	152	406	4050
2	5.20	13.7	151	399	4330
	5.58	14.1	150	401	4240
	4.55	14.3	145	405	4100
	4.45	15.7	149	412	4150
	4.82	15.3	152	405	4200
	4.58	14.3	157	419	4220
3	4.53	14.1	147	403	4310
	4.48	14.0	144	403	4070
	4.21	14.4	148	394	3930
	4.28	13.7	151	394	4080
	4.10	14.9	147	398	3740
	4.6	13.9	150	399	3750
Mean	4.77	14.7	151	407	4110
S.D.	0.42	0.7	4	9	170
%CV	8.7	4.7	2.9	2.3	4.1
%Theoretical	95.4	98.0	100.7	101.8	102.8
%DIFF	−4.6	−2.0	0.7	1.8	2.8
n	18	18	18	18	18

inter-run precision (%CV) ranged from 2.1 and 6.9% ($n = 6$). For QC samples, the inter-run precision and accuracy results from three analytical core runs are summarized in Table 2. The inter-run accuracy (%DIFF) ranged from −4.6% at the LLOQ to 2.8% at the QCH ($n = 18$). The inter-run precision (%CV) ranged from 8.7% at the LLOQ to 2.3% at the QCM ($n = 18$).

3.6. Selectivity

Blank plasma samples from six sources were screened and were found to be free of interference (<20% of the LLOQ peak area and <5% of the LLOQ IS peak area) from endogenous components or other sources at the same mass transitions and retention times as posaconazole and the IS. The method was also sufficiently selective between posazonazole and the IS.

3.7. Dilution integrity

Integrity of dilution was assessed using dilution quality control (QCD) samples prepared at 25,000 ng/mL posaconazole, and diluted 10-fold ($n = 6$). As illustrated in Table 3, the precision and accuracy were 2.0% and −1.2%, respectively.

Table 3
Dilution integrity data for extracted posaconazole samples in human plasma. QCD: dilution quality control. See Table 1 for additional abbreviations. All concentrations are listed to three significant figures.

Sample	QCD 25,000 ng/mL
	2.43E+04
	2.50E+04
	2.43E+04
	2.55E+04
	2.48E+04
	2.43E+04
Mean	2.47E+04
S.D.	500
%CV	2.0
%Theoretical	98.8
%DIFF	−1.2
n	6

3.8. Recovery

The recovery of posaconazole was determined by comparing the peak area responses of extracted QC samples ($n = 6$) to the peak area responses of neat solutions at the same concentrations ($n = 6$). Mean absolute recoveries were 83.4%, 90.9%, and 90.4% for QCL, QCM, and QCH samples, respectively (Table 4).

4. Applications in routine bioanalysis

The bioanalytical method described herein has been used to support routine bioanalysis of clinical samples from numerous posaconazole clinical studies conducted by Schering-Plough Research Institute. As one could anticipate from clinical trials using diverse patient populations, the plasma samples from these patients varied widely in lipid content among other endogenous substances. It was critical, therefore, to ensure that the method performed in a robust, accurate, and reproducible manner in the presence of these potential negative matrix effects in order to develop an efficacious dose strategy across diverse patient populations.

The chromatographic ruggedness can be evaluated by monitoring analyte retention times over the course of several runs. Fig. 5A is a composite plot of posaconazole retention times measured over 14 separate analytical runs of clinical study samples with 4 separate columns and 2 separate UPLC systems. Retention times ranged over a narrow window of 0.56–0.58 min with intra-run and inter-run precisions of 0.2% ($n = 176$) and 1.6% ($n = 4$ columns), respectively. Acceptable chromatographic peak shape was maintained for at least 1200 injections (Fig. 5B). Taken together, these results demonstrate that the fused-core silica columns are sufficiently rugged and robust

Table 4
Recovery results for extracted posaconazole samples compared to neat samples. All peak areas are listed to three significant figures.

Posaconazole recovery	QCs vs. neat					
Sample	QCL neat	QCL	QCM neat	QCM	QCH neat	QCH
Nominal concentration (ng/mL)	15.0	15.0	400	400	4000	4000
Peak area	5.51E+03	4.08E+03	1.31E+05	1.28E+05	1.24E+06	1.19E+06
	5.04E+03	4.43E+03	1.42E+05	1.22E+05	1.30E+06	1.18E+06
	5.46E+03	4.37E+03	1.31E+05	1.17E+05	1.28E+06	1.23E+06
	5.45E+03	4.71E+03	1.34E+05	1.19E+05	1.27E+06	1.15E+06
	5.37E+03	4.52E+03	1.37E+05	1.17E+05	1.37E+06	1.17E+06
	5.72E+03	5.05E+03	1.19E+05	1.19E+05	1.33E+06	1.12E+06
Mean	5.43E+03	4.53E+03	1.32E+05	1.20E+05	1.30E+06	1.17E+06
n	6	6	6	6	6	6
%Recovery	N/A	83.4	N/A	90.9	N/A	90.4

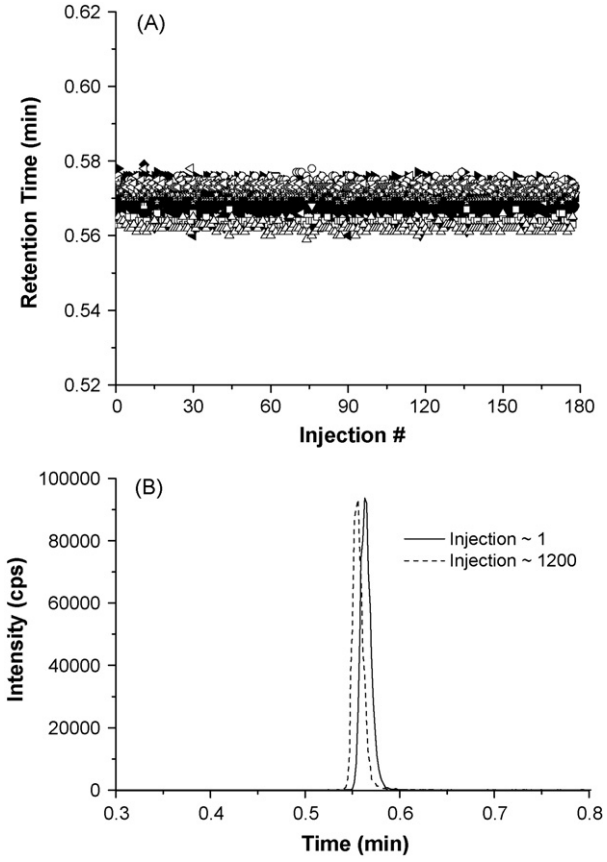

Fig. 5. Halo C18 column performance during routine sample analysis. (A) Retention time of extracted posaconazole samples during 14 analytical runs of ~176 samples each. Data was acquired from four different analytical columns. The bioanalytical method states 0.52 and 0.62 min as the lower and upper retention time limits, respectively. (B) MRM chromatograms of an extracted posaconazole sample (IS traces are shown, m/z 705 → m/z 687) on the ~1st and ~1200th injection on the same Halo C18 column. Injections are overlaid for clarity. Experimental details are listed in Section 2.

for routine bioanalytical analysis of clinical samples. The columns have shown impressive column-to-column reproducibility and can be used for extended periods of time without a loss of performance or an increase in backpressure, being a primary concern with human plasma analysis from clinical studies.

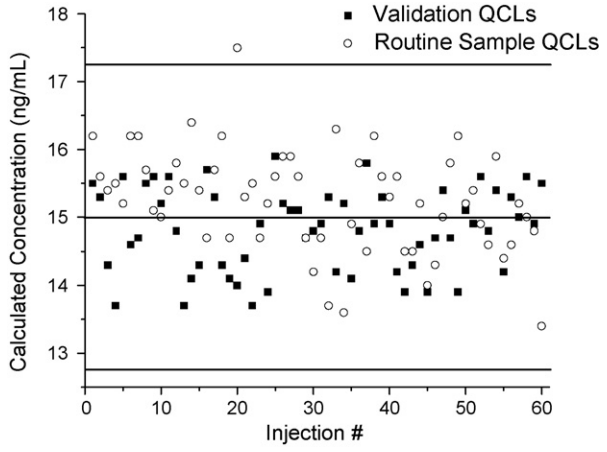

Fig. 6. Calculated concentrations of QCL samples throughout validation and routine sample analysis runs. The upper and lower lines represent ±15% of the nominal concentration (15.0 ng/mL, represented by the middle line). Extraction procedures and LC conditions are listed in Section 2.

Table 5
Incurred sample reanalysis for clinical posaconazole samples. % Difference was calculated with the following equation: % Difference = (Original value − repeat value)/mean of the two values × 100. All concentrations are listed to three significant figures.

Original concentration (ng/mL)	Reassay concentration (ng/mL)	Mean	% Difference
234	250	242	−6.61
22.9	22.9	22.9	0.00
402	358	380	11.6
26.3	25.3	25.8	3.88
312	334	323	−6.81
43.1	44.8	44.0	−3.87
138	128	133	7.52
22.2	22.6	22.4	−1.79
282	277	280	1.79
19.0	18.3	18.7	3.75
135	134	135	0.74
27.6	24.6	26.1	11.5
11.4	11.8	11.6	−3.45
21.5	21.2	21.4	1.41
154	166	160	−7.50
16.3	17.0	16.7	−4.20
372	367	370	1.35
16.9	17.7	17.3	−4.62
17.5	19.1	18.3	−8.74
37.2	35.2	36.2	5.52

Another approach for monitoring method performance during routine sample analysis is to assess QC sample accuracies over the course of several analytical runs and determine bias. Fig. 6 is a plot of calculated QCL concentrations obtained during validation and routine sample analysis runs. Greater than 98% of the 60 QCL samples analyzed during 14 sample analysis runs met the acceptance criteria (Fig. 6). Moreover, the random scatter around the mean concentration suggests that there is no systematic bias being introduced over the course of 14 analytical batch runs.

The FDA has recently recommended that random samples be reanalyzed to ensure method reproducibility, i.e., incurred sample reanalysis (ISR) [34]. Assay reproducibility was assessed with pre-defined acceptance criteria requiring that greater than 2/3 of reanalyzed samples had a relative difference in concentration of ≤20% of the original result. The ISR results from 20 samples covering Cmax and the terminal phase of elimination are presented in Table 5. All samples reanalyzed yielded results that were well within the acceptance criteria further demonstrating that this method is capable of producing reproducible results over time.

5. Conclusions

A mixed-mode cation exchange SPE method was developed and validated for the determination of posaconazole concentrations in human plasma. The application of fused-core silica particle technology permitted substantially higher chromatographic flow rates that afforded a 4-fold reduction in analysis time compared to a previously published assay. The Mac-Mod Halo C18 columns provided excellent column-to-column reproducibility in terms of retention time and peak shape, and may be routinely used for >1000 injections of clinical samples.

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